

Crystals of Cadmium, Zinc Metallothionein

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Single crystals have been grown of Cd,Zn metallothionein isoform II from rat liver. The space group is $P4_12_12(P4_32_12)$ with unit cell dimensions $a = b = 31.0 \text{ \AA}$ and $c = 120.0 \text{ \AA}$, and one molecule in the crystallographic asymmetric unit. The crystals are square bipyramids elongated on the tetragonal c -axis and are grown by repetitive seeding. The crystals are suitable for high resolution structure analysis. Assays of dissolved crystals show that the crystals have the same Cd and Zn content and amino acid composition as the native, as-isolated protein.

Introduction

Metallothioneins have been the subject of intense study in biomedical, physiological, biochemical and genetic experiments, as attested to by this volume. The physical properties of the metallothioneins have been thoroughly studied (1). The mammalian proteins have 61 amino acids, 20 of which are cysteine, and coordinate 7 metals, commonly Zn, Cd or Cu. ^{113}Cd NMR has established that the metals are arranged in two polynuclear clusters of 4 and 3 metals, and has led to models for the clusters as $\text{Cd}_4(\text{cys})_{11}$ and $\text{Cd}_3(\text{cys})_9$ (2). The protein can be cleaved to yield a peptide, α , consisting of the carboxy-terminal 32 residues and containing 4Cd (3). The protein is therefore folded into two domains, one for each cluster, utilizing 9 and 11 cysteines, respectively, for the amino-terminal 3Cd cluster domain and the carboxy-terminal 4Cd cluster domain (4). There is no precedent for these types of metal clusters in known protein structures; however, inorganic analog compounds of Zn and Co are known (5). In this paper we report successful crystallization of rat liver Cd,Zn metallothionein, isoform II, and the properties of the crystals. The crystals are suitable for high resolution X-ray diffraction analysis. A complete sequence determination of isoform II is in progress (6).

Experimental

Cd,Zn metallothionein isoform II (Cd,Zn MT II) was prepared from rat liver as previously described (7,8), except that the material was passed through a second DEAE-cellulose column to insure purity. Fractions from the final G-25 Sephadex column in 0.005 M potassium phosphate, pH 7.8, were pooled and lyophilized. Each sample showed a single band on nondenaturing polyacrylamide electrophoresis gels (Fig. 1). Cd and Zn were determined by atomic absorption spectroscopy. Based on quantitative amino acid analysis the metal content was determined to be 4.0–6.0 mole Cd and 2.5–1.0 mole Zn per mole of protein in eight samples used for crystallization. The amino acid analysis also detected no leucine, histidine, arginine, tyrosine or phenylalanine.

Lyophilized samples are dissolved to make a solution 10 mg/mL in protein containing 1.0 M sodium formate and 0.2 M potassium phosphate at pH 7.5. The solution is equilibrated in 5 to 10 μL volumes against 1.0 mL volumes of 5.0 M sodium formate, pH 7.5, by using vapor diffusion and hanging drops. Within 1 day at 22°C or 3 days at 2°C, several dozen single crystals appear. The larger crystals are used as seeds and transferred into pre-equilibrated droplets of fresh protein solution, just prior to the onset of nucleation. Repetition of the seeding procedure affords large single crystals (9). The crystals are square bipyramidal, morphologically single and highly reflective (Fig. 2).

Variation of the concentrations of sodium for-

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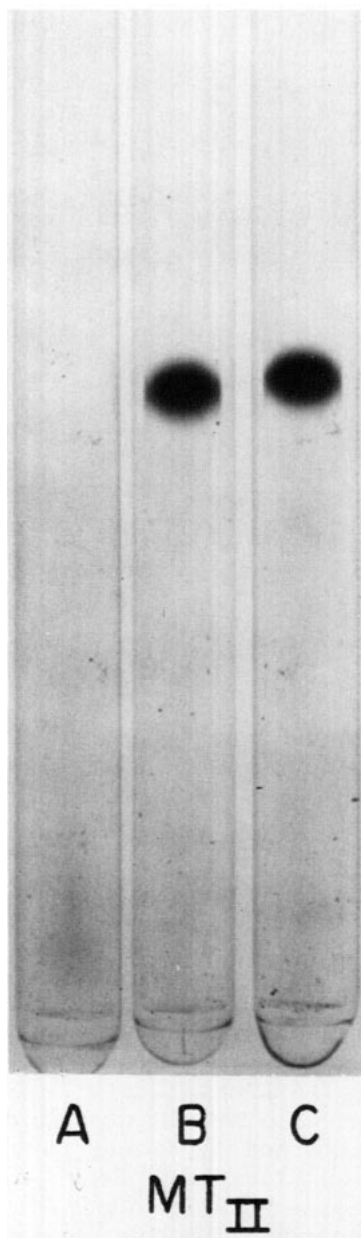


FIGURE 1. Nondenaturing polyacrylamide gel electrophoresis gels of apo-MT (gel A), Cd₇-reconstituted MT (gel B), and native Cd, Zn MT II (gel C). The samples were preincubated with subtilisin (1:30 weight ratio of protease to MT) for 16 hr at 25°C prior to loading 10 µg/gel. The 7.5% polyacrylamide gels were electrophoresed at pH 8.9 and stained with Coomassie blue. The gels are oriented with the anode or tracking dye position at the top.

mate and potassium phosphate in the droplet leads to more rapid nucleation, where literally hundreds of crystals are observed per droplet, or to oiling out or precipitation of the protein. The results of over 650 hanging drop experiments are summarized in a phase diagram (Fig. 3), which is

Table 1. Metal content of Cd, Zn metallothionein isoform II samples and dissolved crystals.

Sample	Metal	As-isolated, g-atom/mole	Dissolved crystals, g-atom/mole
A ^a	Cd	4.0	4.9
	Zn	2.5	2.3
B	Cd	4.3	5.0
	Zn	2.5	2.9

^aThe dissolved crystal sample was desalted with Sephadex G-25 (1.7 × 25 cm column) prior to being assayed.

used as a guide for repetitive seeding. Crystals have been grown with protein from each of eight preparations to date.

Crystals were dissolved and assayed in order to compare their metal content to the as-isolated native protein in low ionic strength solution. Two samples were prepared from two separate batches of crystals. For each, single crystals were washed with 7.0 M sodium formate, collected by centrifugation and dissolved in 0.05 M potassium phosphate, pH 7.8. For both samples the metal ion content was determined by atomic absorption spectroscopy and protein was determined by quantitative amino acid analysis. The data (Table 1) indicate strongly that the crystals contain native Cd,Zn MT II with a full complement of metal ions. Amino acid analysis of the dissolved crystals also showed the same content of lysine, aspartic acid, threonine, glutamic acid, proline, glycine, alanine and valine as the starting samples. Desalted solutions of dissolved crystals also have the same UV absorption spectrum as the as-isolated protein with a distinct shoulder at 250 nm. A fresh protein solution incubated in 1.6 M sodium formate, 0.2 M potassium phosphate, pH 7.5, showed no change in its 250 nm absorption versus 1.6 M sodium formate.

From measuring the volume of several droplets following equilibration and after the onset of nucleation, the protein crystallizes at ~ 2.0 M sodium formate, ~ 0.4 M potassium phosphate. As a further test of these conditions, the ¹¹³Cd NMR spectra of rabbit kidney MT II in 0.8, 1.2 and 1.6 M sodium formate solutions were recorded. The spectra showed no indication of signal loss or line broadening due to facilitated exchange, i.e., displacement of Cd from the protein (I. M. Armitage, private communication).

The density of three crystals, maximum dimensions 0.3 mm, was measured by using a linear density gradient formed by pyridine and chloroform. The crystals were transferred directly from the mother liquor to the gradient with a looped metal wire, as for seeding experiments. The gradient was calibrated with sodium formate solu-

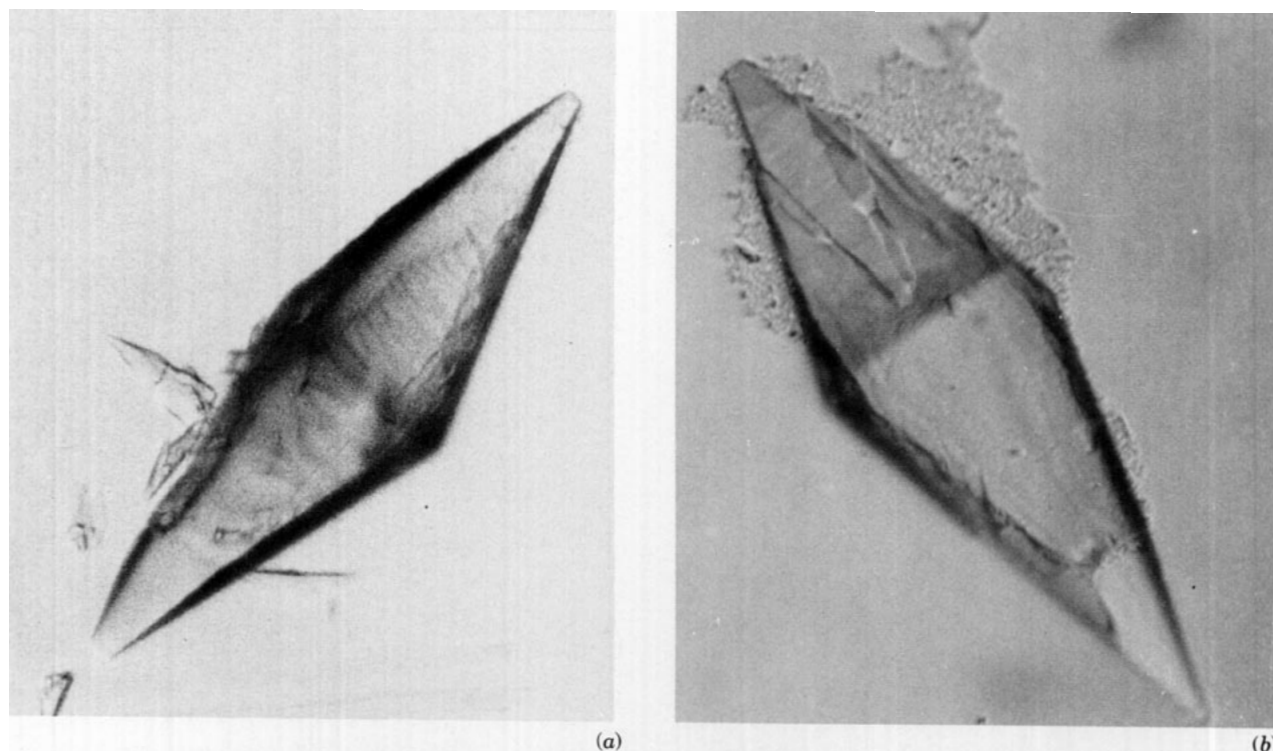


FIGURE 2. Tetragonal single crystals of Cd, Zn metallothionein isoform II. The tetragonal unique axis c is along the long dimension of the crystals. Large single crystals are approximately $0.2 \times 0.2 \times 0.8$ mm in size along the $a \times b \times c$ directions. The outline of the seed crystal is visible in (a). Crystals of either habit exhibit the same diffraction pattern.

tions. For the three crystals the crystal density is 1.29 ± 0.02 g/cm³.

For X-ray experiments, crystals were mounted in 0.7 mm glass capillaries using 7.0 M sodium formate, 0.2 M potassium phosphate, pH 7.5, as a synthetic mother liquor.

Results and Discussion

Figure 4 shows the $0kl$ zone of the diffraction pattern for crystals of Cd,Zn metallothionein isoform II. Reflections are absent for $00l$, $l \neq 4n$, and $0k0$, $k \neq 2n$. The diffraction symmetry is mm for the $0kl$, $h0l$ and hhl zones. The intensity distribution for the $0kl$ and $h0l$ zones is identical, where these films are obtained from the same crystal rotated 90° . The $hk0$ zone shows $4mm$ symmetry. For the $hk0$ and $h0l$ zones, reflections are absent for $h00$, $h \neq 2n$. The Laue group is therefore $4/mmm$ and the space group is $P4_12_12$, or its enantiomer, $P4_32_12$. Unit cell dimensions measured from precession photographs are $a = b = 31.0$ Å and $c = 120.0$ Å. The crystal morphology is a square bipyramid elongated on the tetragonal c -axis.

The crystal density may be calculated from the unit cell parameters (Table 2) by assuming the

density of the mother liquor is 1.08 g/cm³ (2.0 M sodium formate), and by letting $v = 0.64$ mL/g. For one molecule in the asymmetric unit the calculated density is 1.31 g/cm³, in agreement with the observed value of 1.29 ± 0.02 g/cm³. For two molecules in the asymmetric unit, the calculated density is 1.55 g/cm³, well outside the experimental error. If the solvent content of the crys-

Table 2. Crystal data for Cd, Zn metallothionein isoform II.

Characteristic	
Crystal system	Tetragonal
Space group	$P4_12_12$ or $P4_32_12$
Unit cell dimensions	$a = 31.0$ Å
	$b = 31.0$ Å
	$c = 120.0$ Å
	$\alpha = \beta = \gamma = 90^\circ$
Unit cell volume	$115,300$ Å ³
Molecules/unit cell	8
Molecules/asymmetric unit	1
Observed density	1.29 g/cm ³
Calculated density	1.31 g/cm ³
Matthew's coefficient ^a	2.2 Å ³ /dalton ^b
Solvent fraction ^c	0.51
Typical size of crystals	$0.2 \times 0.2 \times 0.8$ mm

^aData of Matthews (14).

^bFor $M_r = 6500$.

^cFor $v = 0.64$ mL/g.

tals is taken as pure water, the calculated density is 1.29 g/cm³. Therefore, the assumed salt content of the mother liquor does not significantly affect the calculated value of the crystal density.

The diffraction pattern shown is reproducible, having been recorded from all the crystals examined to date, and crystals have been grown using eight separate preparations of the protein. Because reflections are observed to at least 1.9 Å (Fig. 5) using as an X-ray source a standard focus sealed tube operated at 35 kv, 15 ma, it is anticipated that even higher resolution data will be observed with a more intense X-ray source. The crystals are stable in the X-ray beam; the diffraction pattern shown in Figure 4 is still observed after five days of exposure to the crystal at 2°C. The crystals therefore are suitable for a high resolution structure determination.

Several facts may be enumerated that argue that the crystals are protein and contain metallothionein in a native state. The diffraction pattern is typical of protein crystals. The crystals extinguish polarized light in a manner consistent with their 422 morphological symmetry, and their color in polarized light (blue-gray) is typical of protein crystals. The crystal growth from the seed often exhibits polarity, consistent with the enantiomorphic space group. Crystalline suspensions examined in an electron microscope reveal square and pyramidal shaped microcrystals of dimensions 1000–2000 Å whose appearance is that expected for a material with a large unit cell repeat, i.e., their surfaces are rough and the edges are ragged, unlike a salt. Large changes in the ionic strength of the mother liquor causes the crystals to shatter, or dissolve, typical of protein crystals. Crystals crosslinked in glutaraldehyde (10) are insoluble in pure water, and colored compounds diffuse into these crystals in a period of hours. The solvent content, consistent with the observed density and molecular weight (Table 2), is also typical of protein crystals.

Dissolved crystals assay reproducibly, within experimental error, for the same Cd and Zn content and amino acid composition as the starting material (Table 1). Dissolved crystals also show the same UV absorption spectrum as the as-isolated protein. The precipitant, sodium formate is not deleterious, as concentrations up to 1.6 M in potassium phosphate buffer have no effect on the UV spectrum or on the ¹¹³Cd NMR spectrum of rabbit liver metallothionein (I. M. Armitage, private communication). As the protein crystallizes from about 2.0 M sodium formate following equilibrium, it is assumed that 1.6 M sodium formate in buffer is a good approximation of the conditions

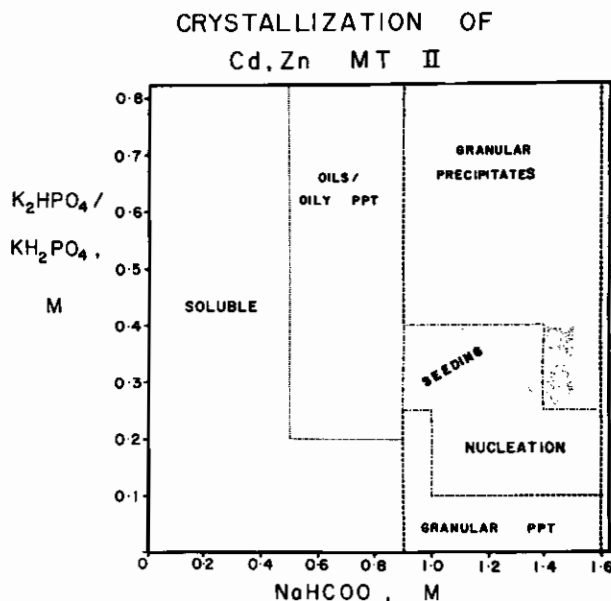


FIGURE 3. Phase diagram for Cd, Zn metallothionein isoform II summarizing results of crystallization experiments in which the concentrations of sodium formate and potassium phosphate, pH 7.5, were varied. The concentrations plotted are those in the protein solution prior to equilibration against a 1.0 mL reservoir solution. The reservoirs contained sodium formate at a concentration five times that in the droplet. The starting protein concentration was 10 mg/mL throughout. Drop volume (5 or 10 μ L) and temperature (2°C or 22°C) affected the rates of equilibration but not the final state of Cd, Zn MT II obtained.

in the crystal. In a related experiment, a sample of rat liver MT II reconstituted with Co (11) formed a dark green oil when equilibrated anaerobically with sodium formate, demonstrating that the oil phase (Fig. 3) does indeed contain protein. Protein precipitation and crystallization from an oil phase has been documented for yeast triose phosphate isomerase (12). At least two other proteins have been crystallized by use of sodium formate: bee venom melittin (13) and *Azotobacter* flavodoxin (unpublished results).

Figure 5 shows the 1kl zone of the reciprocal lattice to 1.9 Å resolution. In the presence of anomalous scattering the true symmetry of this upper level precession photography should be 2, the point group of the crystal being 422. Because the symmetry of the Laue group, 4/mmm, is not strictly obeyed, the mirror plane perpendicular to the *c** direction is broken, and Bijvoet differences are observed, as indicated in Figure 5. These must arise from anomalous scattering in the crystal. For CuK α radiation, $\lambda = 1.54$ Å, the anomalous scattering factors, *f*'', for Cd, Zn and S are 5.0, 0.8 and 0.6 electrons, respectively. The dominant effect, therefore, is from the 5Cd in protein (40Cd

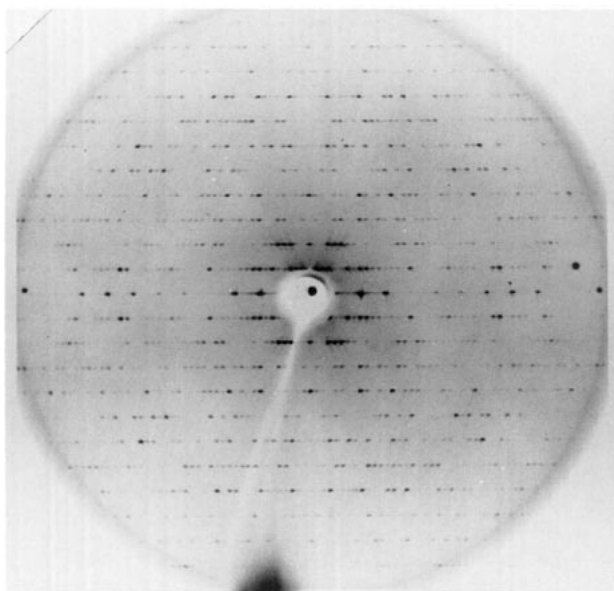


FIGURE 4. X-ray precession photograph of the $0kl$ zone of a tetragonal single crystal of Cd, Zn metallothionein isoform II. The c^* direction is horizontal, b^* is vertical. A 34-hr exposure at 2°C with Ni-filtered CuK_α radiation from a standard focus sealed tube operated at 35 kV, 15 ma. For this film, $\mu = 18^\circ$ ($d_{\min} = 2.5 \text{ \AA}$ resolution).

per unit cell), and this demonstrates the presence of Cd in these protein crystals. Accurate measurement of these subtle, but significant differences, is a means of deriving phase angles. The structure of crambin, a 5000 dalton protein, has been solved by using just the anomalous scattering due to sulfur (15).

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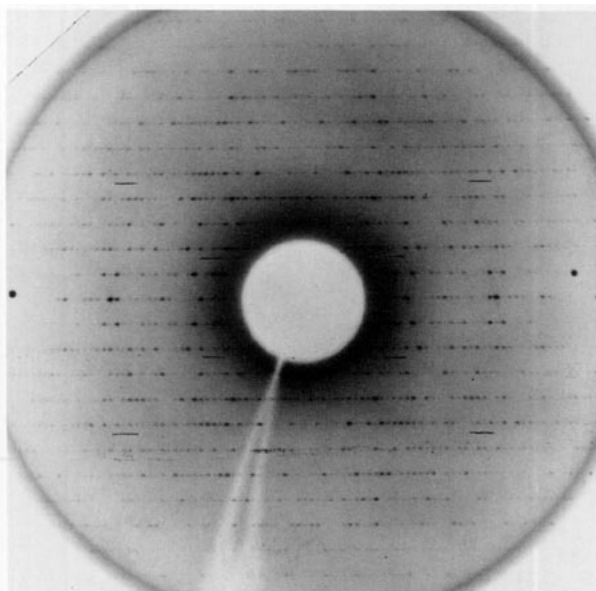


FIGURE 5. Upper level precession photograph of the $1kl$ zone for the same crystal as shown in Fig. 4 under the same experimental conditions. For this film, $d_{\min} = 1.9 \text{ \AA}$ resolution. The symmetry of the zone is 2, because reflections on opposite sides of the vertical pseudo-mirror plane are not exactly equal due to anomalous scattering from Cd ($1kl$, $1kl$ reflections). This is indicated for several reflections on the film.

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